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Bacterial Adenosine 5′-Triphosphate Synthase (F_1F_0): Purification and Reconstitution of F_0 Complexes and Biochemical and Functional Characterization of Their Subunits

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INTRODUCTION

H^+-translocating adenosine 5′-triphosphate (ATP) synthases are multisubunit enzymes which have been found in the cytoplasmic membranes of a variety of bacteria as well as in the inner membrane of mitochondria and the thylakoid membrane of chloroplasts (143). These enzymes can utilize the electrochemical gradient of H^+ built up by either respiration or light for the synthesis of ATP from adenosine 5′-diphosphate and inorganic phosphate (P_i). In bacteria growing under anaerobic conditions, the enzyme functions in the reverse direction, generating an electrochemical gradient of H^+ by the hydrolysis of ATP. In vitro, all enzymes studied so far have a distinct ATP hydrolase activity. ATP synthases from different sources have in common their overall structure; the enzyme consists of two parts: the membrane-peripheral F_1 portion and the membrane-integral F_0 complex (Fig. 1). The F_1 portion carries the catalytic domains of the enzyme and is composed of five different subunits designated α, β, γ, δ, and ε (reviewed in reference 213). There is now convincing evidence that the stoichiometry of these subunits is 3:3:1:1:1 regardless of the organism (21, 51, 94, 114, 203). F_1 is bound to the membrane by interaction with F_0, and in most cases it is easily released into the medium by treatment with either ethylenediaminetetraacetic acid (EDTA) at low ionic strength (130) or chloroform (106). Purified F_1 has been the object of intensive research on the mechanism of ATP hydrolysis (143). In the case of bacteria, the F_0 complex contains at most three different subunits (*a*, *b*, and *c* or χ, Ψ, and ω), whereas in chloroplasts and mammalian mitochondria four to six or even more polypeptides are present (71, 150, 166). At least for the bacterial system, the stoichiometry has been proposed to be $a_1\ b_2\ c_{6-12}$ (51, 94, 215). F_0 is thought to function as an H^+ channel that translocates H^+ across the membrane. This activity can be blocked by the specific inhibitor *N,N′*-dicyclohexylcarbodiimide (DCCD) as well as by various antibiotics, including oligomycin and venturicidin (105, 148). In all cases the site of action of DCCD is subunit *c*, also called proteolipid or DCCD-binding protein (177). DCCD binds covalently to an acidic residue (Asp in *Escherichia coli*, Glu in all other organisms examined so far), which is located within an extended stretch of hydrophobic amino acids. It is therefore tempting to speculate that this carboxyl group might play a crucial role in the H^+-translocating process.

Preparations of F_1F_0 ATP synthases have been reported from several bacteria, including the thermophilic PS 3 (189), *E. coli* (50, 56), *Streptococcus faecalis* (104), *Mycobacterium phlei* (103), *Rhodospirillum rubrum* (12, 171), *Micrococcus luteus* (163), *Synechococcus* sp. strain 6716 (111), *Wolinella succinogenes* (17), and *Clostridium pasteurianum* (26). The enzyme from the latter organism has been reported to contain only three F_1 and one F_0 subunits. It has been postulated (119) that this enzyme represents an "ancient version" of F_1F_0 adenosine triphosphatases (ATPases) as may have occurred early in evolution. The

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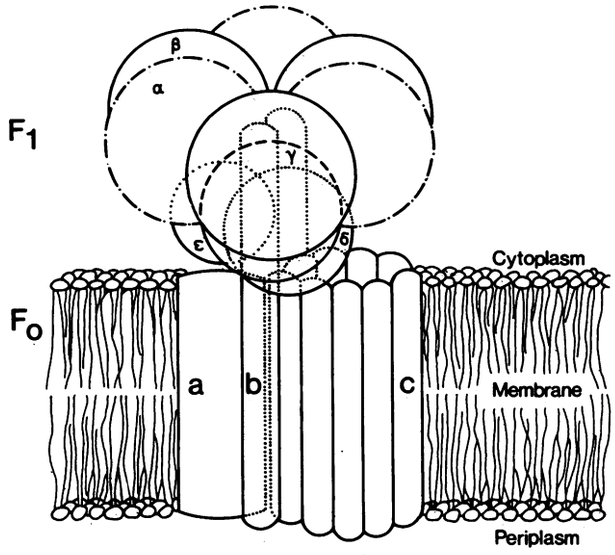

FIG. 1. Schematic representation of *E. coli* ATP synthase (F_1F_0). This model is not drawn to scale. For further details, see section, "Open Questions, Perspectives, and Models."

enzymes from PS 3 and *E. coli* have been characterized most extensively, the latter also by genetic approaches (reviewed in references 59, 63, and 218).

In *E. coli* the genes coding for the subunits of the synthase are arranged in an operon located in the region designated *unc* (*pap* or *atp*) at 84 min on the *E. coli* chromosome (12) (Fig. 2). The complete nucleotide sequence of the entire operon has been established (for review, see reference 221). Surprisingly, in the case of two photosynthetic bacteria only the genes for the F_1 subunits are clustered in an operon, whereas the genes for the F_0 subunits have not been identified so far (42, 206). In eucaryotic organisms the subunits are encoded by nuclear as well as organellar genes (135, 209). In chloroplasts and in the cyanobacterium *Synechococcus* (30, 72), the gene order for the F_0 subunits remains the same as in *E. coli*.

A number of reviews covering different aspects of the ATP synthase (biochemistry of F_1F_0 and F_1: 1, 7, 46, 59, 71, 92, 180, 181, 198, 213; structure of F_0: 80, 82, 166, 177, 179; genetics: 63, 218) as well as a book (143) have been published recently. Rather than summarizing these data again, we present here a critical review on different F_0 preparations and their capacity to translocate H^+. Besides some methodological aspects, this article deals with dissociation and reassembly experiments and what we can learn from them about the role of the single subunits in a functional F_0 complex. Finally, a comparison of data reported for bacterial and eucaryotic systems leads us to a general discussion of the mechanism of H^+ translocation through F_0.

CRITERIA FOR FUNCTIONAL F_0

Passive Proton Translocation

F_0, as an integral membrane protein, can only be solubilized and purified in the presence of detergents. Since this inevitably abolishes vectorial H^+ transport through the protein, a lipid bilayer should be provided allowing the reinsertion of the complex with restoration of vectorial functions (41, 154). This process is called "reconstitution" throughout this article, and it should be clearly distinguished from the reassembly of enzyme complexes from their constituent subunits. Also, it should be emphasized that reconstitution is not an approach to mimic physiological conditions. Its applications are limited to assaying the purification of membrane proteins, analyzing the mechanism of the catalytic process, and identifying the participating components (154).

A suitable housing facility for purified membrane proteins is provided by phospholipid vesicles, called liposomes, which are closed lipid structures providing both a membrane matrix and an internal volume separated from the medium (41). Thanks to the pioneering work of E. Racker, a variety of methods of reconstitution into liposomes are now available (154). In 1962, Mueller et al. (127) introduced the planar "black" lipid membrane as a model system to study the molecular basis of transport in biochemical membranes. The rationale for using planar bilayers in membrane reconstitu-

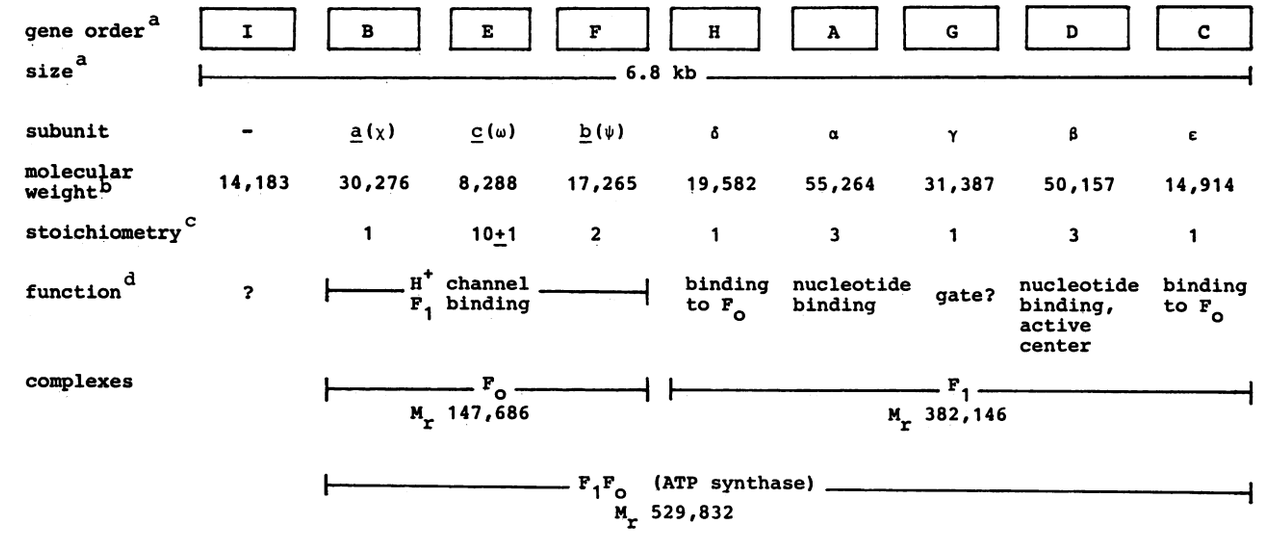

FIG. 2. Relationship between genes and subunits of *E. coli* ATP synthase. [a]Taken from reference 68; [b]taken from reference 181; [c]taken from reference 51; [d]taken from reference 218. kb, Kilobases.

tion studies involving ion channels and pumps resides in the possibility of characterizing unit electrical events (123).

After reconstitution, F_0 preparations have to meet at least two criteria to be considered functional: (i) they should translocate H^+, and (ii) they should bind F_1 in such a way that this H^+ translocation is coupled to the enzymatic activity of F_1. To test for the first criterion, membrane vesicles or liposomes containing F_0 are loaded with K^+. Efflux of K^+ is elicited by the addition of valinomycin, thereby generating an electrical potential ($\Delta\Psi$) across the membrane, which in turn drives protons back into the lumen of the vesicle (6). An oppositely oriented K^+ diffusion potential can be imposed by bathing Na^+-loaded vesicles in a sucrose solution with K_2SO_4 added externally. In this case, protons are driven out of the lumen of the vesicles (133).

If the initial rate of H^+ flux is sufficiently slow, it can be estimated reasonably well from the initial slope of the pH trace (46). The buffer-loaded vesicles are suspended in a minimally buffered medium, and proton fluxes from or to the suspending medium are recorded with a pH electrode. Care should be exercised in demonstrating that the initial rate of H^+ translocation is linearly related to the amount of F_0 incorporated into the liposomes and the magnitude of $\Delta\Psi$. Alternatively, the rate of proton influx into vesicles can be measured by the rate of fluorescence quenching of acridine dyes, e.g., 9-aminoacridine or 9-amino-6-chloro-2-methoxy-acridine. These compounds are thought to accumulate in vesicles in response to a ΔpH, interior acidic. If equilibration of the dye occurs rapidly, and if certain other criteria are met (174), then proton influx in response to an artificially imposed $\Delta\Psi$ (interior negative) should be related to fluorescence quenching. Although this method suffers from more assumptions than the other one and no initial rate of H^+ uptake can be calculated from the data obtained, it is considerably more convenient.

Interaction with F_1

The second criterion for a functional F_0 is that it should bind F_1 so that its ATPase activity is coupled to H^+ translocation. This can be checked by the following methods. If purified F_0 is reconstituted into liposomes with an inside-out orientation, addition of F_1 leads to a functional F_1F_0 complex. Addition of ATP to the medium results in the hydrolysis of this compound, which is coupled to the translocation of H^+ into the lumen of the vesicles. This process can be monitored by simply measuring DCCD-sensitive ATPase activity. It should be mentioned that the ATPase reaction per se does not require closed vesicles. The latter are, however, necessary for monitoring the proton-translocating activity of F_0 (for instance by the fluorescence quenching of 9-amino-6-chloro-2-methoxy-acridine). This combined assay has its limitations, and care should be exercised in running proper controls (46).

Alternatively, the ATP-$^{32}P_i$ exchange activity (153) of a reconstituted F_1F_0 complex may be used as a measure for F_0 function. The rationale for this is that ATP-$^{32}P_i$ exchange, as can readily be seen from a chemiosmotic formulation of the reaction, involves the translocation of protons via the membrane-bound ATP synthase in both directions. However, the relative activities of the synthesis and the hydrolysis reaction may vary greatly with the reconstitution method selected and the purification procedures applied. It is, therefore, not surprising that the ATP-$^{32}P_i$ exchange reaction does not always reflect the properties of the overall system (153).

Another approach to test the function of the reconstituted F_0 complex lies in the measurement of ATP synthesis after rebinding of F_1 and artificial imposition of $\Delta\Psi$ and ΔpH. Alternatively, F_1F_0 and bacteriorhodopsin can be co-reconstituted together into liposomes and the light-dependent phosphorylation can be measured (153, 211). Since two reconstitution steps with different conditions are used with this method, difficulties have been experienced in maintaining optimal activities for both enzymes. The latter is also true for the assay in which F_0 and bacteriorhodopsin have been reconstituted into the same liposome (3). Upon illumination, the electrochemical gradient generated by the pumping activity of bacteriorhodopsin is dissipated by the proton flux through F_0. The electrochemical gradient is restored by the addition of DCCD.

BACTERIAL F_0 COMPLEXES

Preparations from PS 3 and *E. coli*

In 1977, Kagawa and co-workers were the first to report the purification of an F_0 complex from the thermophilic bacterium PS 3 (139). They obtained their F_0 preparation by treatment of the isolated ATP synthase (TF_1F_0) with high concentrations of urea. TF_0 precipitated from the solution after several hours at low temperature and was simply collected by centrifugation. With a slightly modified procedure, TF_0 was obtained almost quantitatively (190).

The isolation of an F_0 complex from *E. coli* was not reported until 1980 (133, 164). This delay was mainly because a suitable procedure to prepare ATP synthase in sufficient amounts was not at hand. In 1979, Foster and Fillingame (50) and Friedl et al. (56) independently published protocols to prepare F_1F_0. As with PS 3, chaotropic reagents were then applied (urea [164]; $KClO_4$ [58]) to destroy the hydrophobic interactions between F_1 and F_0 and to precipitate the F_0 complex. Negrin et al. (133) used a somewhat different approach. They reconstituted purified F_1F_0 into liposomes, partially removed F_1 by treatment with EDTA, and finally, collected the proteoliposomes by centrifugation. This procedure resulted in the enrichment (50 to 60%) of F_0 in the pellet.

The major drawback of all of these protocols was their low yield in protein. This changed when a new hydrophobic gel material became available (31). This resin contains deoxycholic acid molecules as the active ligands, which are covalently linked to agarose beads via poly-L-lysine. Briefly, purified F_1F_0 was bound to the resin (presumably via F_0) and dissociated by urea, and F_1 was eluted from the column. Finally, F_0 was collected by elution with a detergent-containing buffer. The protein yield in F_0 corresponds to 50 to 60% of the total F_0 present in the bound F_1F_0 complex (165; for a detailed description of the procedure, see reference 170). Most recently, F_0 has been isolated directly from F_1-stripped membranes of *E. coli* (167), which is even more efficient and less time-consuming. For this procedure, an *E. coli* strain has been used which overproduces the enzyme severalfold, due to the presence of a transducing λ bacteriophage that carries the entire *unc* operon (52, 122).

Brodie and co-workers (27, 103) reported the purification of F_1F_0 and F_0 from *M. phlei*. In this case, F_0 was dissociated from F_1F_0 bound to adenosine 5'-diphosphate–Sepharose by omitting KCl from the equilibration buffer. A summary of the various F_0 preparations is given in Table 1.

Subunit Composition

The F_1F_0 preparations reported so far contain a total of, at most, eight different subunits, five of which belong to the F_1

TABLE 1. F_0 preparations from bacteria, mitochondria, and chloroplasts

Source	Purification procedure	Yield	Reconstitution method	Phospholipid(s) used[a]	Assays[b]	Reference(s)
Bacteria						
PS 3	Dissociation of TF_1F_0 (urea)	High	Detergent dialysis	PS 3	C, D, E	139
E. coli	Dissociation of F_1F_0 (urea)	Low	Detergent dialysis	Soybean	E	164
E. coli	Hydrophobic interaction chromatography	High	Detergent dialysis; freeze/thaw/sonication	Soybean; PE/PC/PS	A, B, D	165, 169
E. coli	Extraction of F_1-depleted membranes, ammonium sulfate precipitation	High	Detergent dialysis	PE/PC/PS	A, D	167
E. coli	EDTA treatment of F_1F_0 liposomes	Low	Freeze/thaw/sonication/dialysis	*E. coli*	D	133
E. coli	Dissociation of F_1F_0 ($KClO_4$)	Low	Detergent dialysis; incubation	Soybean	B, D, E	58
M. phlei	Dissociation of F_1F_0 at ADP-sepharose (removal of KCl)[c]	Low?	Incubation	Soybean	A, F	27
Mitochondria						
Beef heart	Dissociation of F_1F_0 (NaBr)	?	Detergent dialysis	Soybean	A, D	64
Beef heart	Dissociation of F_1F_0 (NaBr)	?	Sonication	?	D	184
Beef heart	Dissociation of F_1F_0 (NaBr)	?	Freeze/thaw/sonication	Asolectin	E	160
Beef heart	Dissociation of F_1F_0 (NaBr)	?	Sonication	Soybean	A, C	61
Beef heart	Extraction from submitochondrial particles, ammonium sulfate precipitation	High	Incubation; freeze/thaw/sonication	PE/PC	A, C, G, H	3
Chloroplasts						
Lettuce	Extraction of CF_1-deficient subchloroplast particles, sucrose gradient, hydrophobic interaction chromatography	High	Detergent dilution + freeze/thaw	Asolectin	G	99

[a] PE, Phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine.

[b] A, DCCD-sensitive reconstituted F_1F_0-ATPase activity; B, ATP-dependent acridine-dye fluorescence quenching; C, ATP-P_i exchange; D, passive H^+ uptake/release (pH electrode); E, passive H^+ uptake/release (acridine dye); F, oxidative phosphorylation; G, ATP synthesis (plus bacteriorhodopsin); H, H^+ leakage. Methods A, B, C, F, and G require reconstitution with F_1 first.

[c] ADP, Adenosine 5'-diphosphate.

portion of the enzyme (α to ε). The remaining three polypeptides were considered to be constituents of F_0. In *E. coli* they have been designated either *a*, *b*, *c* (56) or χ, Ψ, ω (50).

The subunits of TF_0 from PS 3 were first reported from sodium dodecyl sulfate (SDS) gels to have apparent molecular weights of 19,000, 13,500, and 5,400, respectively (189). For the smallest subunit, which is also known as DCCD-binding protein, the exact molecular weight has been calculated from the amino acid sequence to be 7,310 (79). In addition to the three polypeptides, bands with apparent molecular weights of >19,000 are present in the preparation (139), which might, in part, be residual amounts of TF_1 subunits.

For *E. coli*, the apparent molecular weights of the F_0 subunits were first reported by Foster and Fillingame to be 24,000, 19,000, and 8,000, respectively (deduced from an SDS gel of their F_1F_0 preparation [50]). Indeed, polypeptides with such apparent molecular weights can be identified in all F_0 preparations reported so far (58, 133, 164, 165, 167). The preparations differ, however, with respect to contaminating proteins. Strong support for the notion that the three polypeptides discussed above were the true subunits of F_0 came from two lines of evidence. (i) Gibson and co-workers (reviewed in reference 63) demonstrated the existence of three F_0 genes and identified their respective products. The latter turned out to have apparent molecular weights close to those determined for the polypeptides present in F_0 preparations (58, 133, 165). (ii) Miki et al. (122) have described the isolation of a specialized λ phage which transduces chromosomal deoxyribonucleic acid (DNA) of *E. coli* including the genes for the ATP synthase complex. Induction of this phage resulted in the overproduction of each of the eight subunits of the enzyme (52). When the DNA sequences of these genes became available (62, 95, 138), the exact molecular weights of subunits *a* and *b* were deduced to be 30,276 and 17,265, respectively. The exact M_r of subunit *c* (8,288) had been determined earlier from its amino acid sequence (177).

For *M. phlei* and *S. faecalis*, the reported apparent molecular weights of putative F_0 subunits fall into the same range as those from *E. coli*. All data are summarized in Table 2.

Stoichiometry

The TF_0 complex has been reported to be composed of one copy of the 19-kilodalton (kDa) polypeptide (whether this protein is a subunit of TF_0 at all is still questionable; see below), two to three copies of the 13.5-kDa polypeptide, and four to six copies of the DCCD-binding protein. These data were obtained from SDS gels of purified TF_1F_0, prepared from cells which had been grown on ^{14}C-labeled amino acids (94). Based on these data, the entire TF_0 complex would exhibit an apparent M_r of 90 to 100 kDa. However, as the authors pointed out in a recent review (93), the amount of radioactivity incorporated might have been too low to be accurate for the smaller subunits.

For *E. coli*, Foster and Fillingame determined the subunit stoichiometry by isolating the F_1F_0 complex from cells which had been grown in the presence of either [^{14}C]glucose

TABLE 2. Molecular weights of the subunits of bacterial F_0 complexes

Bacteria	Mol wt of given subunit			Reference(s)
	a, χ	*b*, ψ	*c*, ω	
E. coli	30,276[a]	17,265[a]	8,288	58, 133, 165
PS 3	19,000	13,500–15,500	5,400–6,500[b]	139, 189
M. phlei[c]	25,000	18,000	8,000	27
S. faecalis[d]	27,000	15,000	6,000	104

[a] On SDS gels the proteins run with apparent molecular weights of 24,000 and 19,000, respectively.
[b] The correct M_r based on amino acid sequence is 7,310 (79).
[c] To our knowledge, no SDS gel has been published; the authors stated that their F_0 preparation is incompletely dissolved in boiling SDS and an additional 58-kDa protein is seen on SDS gels.
[d] Identified from an F_1F_0 preparation; no F_0 preparation has been reported so far.

or [^{35}S]sulfate (51). After separation of the subunits on SDS gels, they calculated, from the relative amounts of radioactivity incorporated into the individual subunits, a ratio of a_1 b_2 $c_{10 \pm 1}$, leading to a molecular weight of 147,000 for the entire F_0 complex. Thus, a discrepancy exists between the values reported for subunit *c* from PS 3 and *E. coli*. The stoichiometry for the *E. coli* F_1F_0 complex is supported by the analysis of the membrane-bound complex derived from an overproducing strain (51) and has independently been confirmed (215) (in fact, the latter study even reported 12 to 15 copies of subunit *c* per F_0 complex). From functional studies it is evident that the F_1F_0 complexes from PS 3 and *E. coli* must be very similar: the subcomplexes (F_1 and F_0) from both organisms are even interchangeable (191a). We therefore favor the notion that the stoichiometry for the F_0 subunits reported by Foster and Fillingame (51) also applies to PS 3.

In the case of subunit *b*, further support for a dimeric structure has been obtained from cross-linking (10, 73, 176) and chemical modification (169) studies.

Reconstitution

Already in early studies purified TF_0 catalyzed passive H^+ translocation in liposomes as well as "functional" interaction with purified TF_1 (139). Both TF_1F_0-mediated ATPase activity and ATP-$^{32}P_i$ exchange were reported to be sensitive toward DCCD. Furthermore, passive H^+ uptake was blocked by either TF_1 or antibodies raised against TF_0. From these experiments, the initial rate of H^+ uptake was calculated to be 6 $H^+ \times s^{-1} \times TF_0^{-1}$. In this preparation, only 15% of the total TF_0 was active as judged by the binding of TF_1. Taking this into account, the ATP-$^{32}P_i$ exchange catalyzed by the reconstituted complex was comparable to that of intact TF_0F_1. More recently, using a new procedure for preparing K^+-loaded TF_0-containing vesicles, Sone et al. (187) obtained a much higher initial rate of H^+ uptake as well as stoichiometric binding of TF_1. This finding indicated that not the preparation of TF_0 itself, but the original K^+-loading procedure was responsible for the inactivation of TF_0. As described below, this new method has also been successfully used for the *E. coli* F_0 complex (165). Based on a molecular weight of 90,000 for TF_0, the initial rate of H^+ uptake obtained by the new method corresponds to 47 $H^+ \times s^{-1} \times TF_0^{-1}$. Unfortunately, no comparison with native membranes was given by the authors (see Table 3).

All F_0 preparations from *E. coli* exhibited passive H^+ translocation. This activity was measured after generation of a membrane potential by K^+/valinomycin and could be inhibited by DCCD (58, 133, 164, 165, 167, 172). Moreover, it was demonstrated for two preparations that the H^+ translocation could be blocked almost completely by purified F_1 (58, 165, 172). This finding indicates that F_0 had been incorporated into the liposomes with the F_1-binding site(s) exposed exclusively to the medium. As calculated from reference 146 and shown in Table 3, F_1-depleted membranes of *E. coli* exhibited initial rates of H^+ uptake in the range of 113 ng of ions min^{-1} mg^{-1}. Based on a content of 0.105 μmol of F_0 per mg of membrane protein (46), this value corresponds to a flux rate of 18 $H^+ \times s^{-1} \times F_0^{-1}$ (at pH 6.5). The

TABLE 3. Rates of H^+ translocation through F_0 and DCCD-binding proteins from different sources

Source	Initial rate of H^+ uptake/release (ng of ions min^{-1} mg^{-1})	Turnover (H^+ s^{-1} $channel^{-1}$)	Assay conditions [pH; Δψ(mV)]	Reference
Bacteria				
PS 3	4×10^3	6	7.4; 103	139
PS 3	31×10^3	47	7.2; 94	187
E. coli (membranes)	113	18	6.5; ?	46
E. coli	10×10^3–12×10^3	30	6.5; ?	133
E. coli	870	2.2	7.5; ?	58
E. coli	2.6×10^3	6.5	7.2; 94.5	165
E. coli	8.4×10^3	21	7.0; 94.5	169
Mitochondria				
Intact	ND[a]	404	6.2; 100	139, Calculated from reference 74
F_0 (beef heart)	108	0.3	7.7; ?	184
F_0 (beef heart)	2.8×10^3	6.5	7.5; ?	64
DCCD-binding protein (yeasts)	40×10^3	5[b]	7.5; ?	100
DCCD-binding protein (yeasts)	ND	100	7.0; 100	162
Chloroplasts				
Intact (-CF_1)	ND	6.2×10^3	7.8	173
DCCD-binding protein	675	0.09[b]	7.5	185

[a] ND, Not determined.
[b] Per proteolipid molecule.

preparations of Negrin et al. (133) and Schneider and Altendorf (165, 167) exhibited rates which come close to this value (Table 3).

The rate of H^+ translocation appears to be dependent on the lipid composition. Thus, for maximal activity a high content of phosphatidylethanolamine seems to be required (133, 165, 167). This is in fact the predominant phospholipid in the *E. coli* inner membrane (113). Table 3 summarizes the data on H^+ flux through F_0. Although there were differences in the methods used to prepare F_0 and the proteoliposomes as well as in initial pH values and diffusion potentials, the reported values are close to each other. This lends support to the notion that we are in fact dealing with rates of physiological significance.

The F_1-binding activity of F_0 preparations was tested by two groups (58, 165, 167). Both preparations exhibited ATP-dependent, DCCD-sensitive H^+ translocation through F_0 after reconstitution with purified F_1 (58, 165). Also, the reconstituted ATPase activity itself was shown to be DCCD sensitive (167). In this case it was demonstrated that a molar ratio of 1:1 for F_1 and F_0 was optimal for activity.

Considerably less information is available on the F_0 preparation from *M. phlei* (27). DCCD-sensitive ATPase activity was reconstituted from isolated F_0 and F_1. However, the inhibition by 0.6 mM DCCD was only between 22 and 33%. By contrast, the P/O ratio of oxidative phosphorylation, though considerably lower than the value reported earlier for reconstitution of intact F_1F_0 (103), was inhibited to 80% by DCCD. Thus, the coupling of F_1 to F_0 may still be suboptimal in this preparation.

Primary and Secondary Structures

Due to the lack of DNA or protein sequences for the 19- and 13.5-kDa proteins in TF_0, no structural data are available. However, from the amino acid composition of the 13.5-kDa protein a similarity to the *E. coli* subunit *b* can be deduced (191, 192). Both proteins are enriched in charged as well as hydrophobic amino acids. They both are attacked by proteases, resulting in the loss of F_1-binding capacity (73, 76, 147, 149, 190, 193) (see also below). As to the DCCD-binding protein of TF_0, its amino acid sequence has been determined and its homology to the *E. coli* subunit *c* has been established (79, 177). This is further supported by the observation that antibodies raised against *E. coli* subunit *c* cross-react with the DCCD-binding protein from TF_0 (109, 191a). By contrast, no cross-reaction with the TF_0 subunits was obtained with *E. coli* anti-*a* or anti-*b* antibodies (191a).

In the case of *E. coli*, secondary structures for the F_0 subunits have been proposed on the basis of DNA sequencing data (56, 80, 95, 138, 179, 217, 218). Subunit *a* as a whole is a hydrophobic protein which could span the membrane five to seven times, thereby leaving the N-terminal region exposed to the water phase (28, 73, 218). However, experimental evidence for this is still lacking.

Subunit *b* has one hydrophobic segment of about 33 amino acids at the N-terminal region, while the rest (80%) of the molecule is extremely hydrophilic and highly susceptible from the cytoplasmic side of the membrane to a variety of proteases (73, 76, 147). Experimental evidence that the N-terminal region is in contact with the lipid phase stems from studies with 3-trifluoromethyl-3-(*m*-[^{125}I]iodophenyl) diazirine (TID), a carbene-generating reagent that labels amino acid residues exposed to the lipid phase of the membrane (75). It is therefore conceivable that the protein is anchored in the membrane by its N-terminal domain whereas the C-terminal region faces the cytoplasm.

As already mentioned, subunit *c* has been studied extensively. It is even more hydrophobic than subunit *a* and is supposed to contain two membrane-spanning segments connected by a hydrophilic loop (hairpin-like structure [177]). Information on the orientation of the "hairpin" in the membrane has come from the chemical modification of suitable amino acid residues. The amino acid sequence of subunit *c* exhibits two tyrosine residues, one (Tyr-10) close to the N-terminal and the other (Tyr-73) close to the C-terminal end of the polypeptide chain. Both residues can be modified by tetranitromethane. However, in right-side-out vesicles only nitrotyrosine-73 is converted to the amino form by $Na_2S_2O_4$. This indicates that the C-terminal region faces the periplasm. (Preliminary results had indicated [34] that the nitrotyrosine at the N-terminal end became reduced to the amino form. However, detailed analysis of the modified peptides revealed that this was not correct.) Since the nitrotyrosine at the N-terminal end is not reduced to the amino form in either right-side-out or inside-out vesicles, this part of the polypeptide chain is probably buried within the membrane (34a). However, as both the stretch including Tyr-10 and that including Tyr-73 were labeled by the lipid-soluble TID (75) and by the lipophilic tetranitromethane, also the C-terminal tyrosine is probably in close contact with the membrane.

There is genetic as well as immunological evidence that the hydrophilic loop of subunit *c* might be in contact with F_1. Mosher et al. (126) reported on the characterization of an *E. coli* mutant in which Gln-42 is replaced by Glu, leaving the H^+ translocation unimpaired. However, proton flow through F_0 could no longer be blocked by the addition of F_1 and the observed membrane-bound ATPase activity was insensitive to DCCD. Bragg and co-workers (108) described an anti-*c* antibody that blocked H^+ translocation through F_0 and interfered with the binding of F_1 to F_0 in F_1-stripped everted vesicles. They also showed (109) that modification of either methionine or arginine residues of subunit *c* affected binding of the anti-*c* antibodies and F_1, respectively. Since it is highly unlikely that chloroform-methanol-purified subunit *c*, immobilized on microtiter plates (109), binds any appreciable amount of F_1 at all, the physiological relevance of the data presented is questionable (see also below).

For a schematic representation of the topography of the F_0 subunits in the membrane, see Fig. 3. However, it should be noted that an alternative model for subunit *c* has been presented (37). According to this proposal, a third membrane-spanning helix is originated at the invariant proline residue 43, giving rise to a cluster of hydrophilic residues which then can form a proton wire. Interestingly, it has recently been suggested that membrane-buried proline residues are inherent constituents of transport proteins (22, 37).

Role of Specific Amino Acid Residues

To investigate the role of specific amino acid residues in TF_0 functions, Kagawa and co-workers modified liposome-integrated TF_0 with various chemical reagents and subsequently assayed for H^+-translocating and TF_1-binding activities (188, 191). H^+ translocation was inhibited by a water-soluble carbodiimide (at a rather high concentration, in the presence of ethylenediamine). From this the authors concluded that, in addition to Glu-56 of the proteolipid

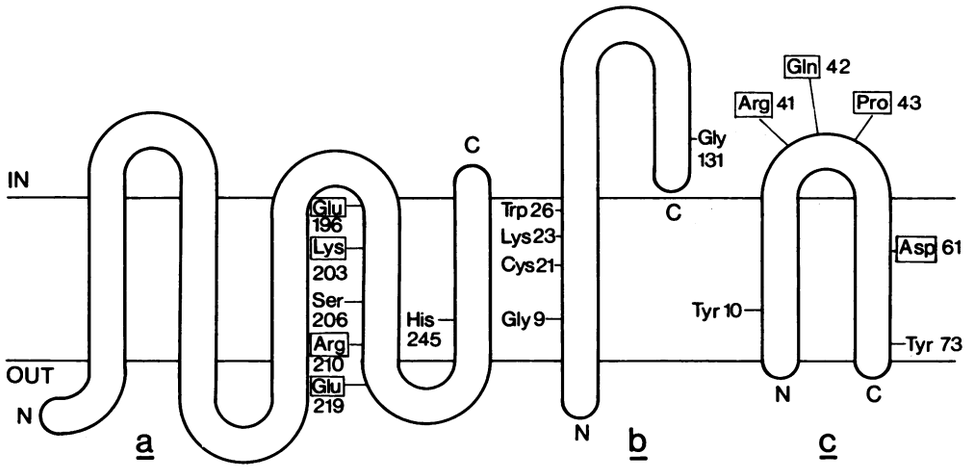

FIG. 3. Subunits *a*, *b*, and *c* from *E. coli* F_0 and their proposed orientation in the membrane (taken from reference 102, modified). Conserved amino acid residues are boxed.

(DCCD-binding site), other carboxyl groups also may be involved in H^+ transport (191).

Tyrosine residues, as judged by the inhibitory effects of tetranitromethane and iodine, were also thought to be required for H^+ translocation. The authors showed by amino acid analysis that complete inhibition of H^+ translocation by tetranitromethane was accompanied by the modification of one-third of the tyrosine residues present in the proteolipid (188).

Modification of TF_0 liposomes with phenylglyoxal, a reagent known to react specifically with arginine residues, resulted in the inhibition of both H^+ translocation and TF_1 binding. Again, rather high concentrations of the modifying compound had to be used to observe 80 to 90% inhibition of both functions. Since TF_1 binding was more rapidly affected by the reagent than H^+ translocation, the authors concluded that two different inhibition sites were involved, one in the 13.5-kDa protein and the other in the DCCD-binding protein. For the latter subunit, a decrease in the amount of arginine residues caused by the treatment of TF_0 liposomes with phenylglyoxal was inferred from amino acid analysis (188).

From these studies, the overall conclusion was drawn that all three groups of residues in subunit *c* are participating in the H^+-translocating process, whereas arginine residues in the 13.5-kDa protein are involved in the interaction with TF_1. However, it should be noted that direct evidence (e.g., by radiolabeling) for the exclusive modification of the proteolipid by the various reagents is still lacking. Furthermore, the 13.5-kDa protein also contains all three groups of residues (191), and on the basis of the evidence available, it is premature to exclude that the 13.5-kDa protein also may be involved in H^+ translocation. Finally, the TF_0 preparation used for these studies still contained the 19-kDa protein and additional polypeptides, the role of which as contaminants or true constituents of TF_0 has still to be established (see next section).

Similar experiments have been carried out with *E. coli* F_0 liposomes (194). Also in this case, H^+ translocation was inhibited by modification of carboxyl residues with water-soluble carbodiimides. Interestingly, it has been shown that inhibition of H^+ translocation in F_1-depleted everted membrane vesicles and F_0-containing liposomes by a water-soluble carbodiimide is probably caused by cross-linking of the C-terminal carboxyl group of subunit *c* to phosphatidylethanolamine (110). It is not known yet whether F_0 functions are affected by water-soluble carbodiimides in the absence of phosphatidylethanolamine. However, those experiments may be hampered by the obvious requirement for this phospholipid in H^+ translocation (see above).

Also, diethylpyrocarbonate, a reagent specific for histidine residues, inhibits H^+ translocation through *E. coli* F_0 (194). As there is no histidine residue in subunit *c* and only one in subunit *b*, located in the hydrophilic part, the inhibition of proton translocation by diethylpyrocarbonate might indicate the involvement of histidine residues in subunit *a* (a total of seven, at least two of them suggested to be located within the membrane) in that process. This would point to a more direct role of subunit *a* in the translocation of H^+ (see next section).

Treatment of F_0 liposomes with either phenylglyoxal (194) or 2,3-butanedione (6a) resulted in the inhibition of F_1-binding activity. Passive H^+ uptake was only slightly inhibited by phenylglyoxal but even enhanced by 2,3-butanedione. Labeling of F_0 liposomes with [^{14}C]phenylglyoxal resulted in the incorporation of radioactivity in all three subunits. However, subunit *b* became labeled much faster than subunits *a* and *c*. In the presence of F_1, subunit *b* was partially protected against the label (6a). From these data the authors concluded that predominantly arginine residues in subunit *b* are involved in the interaction with F_1. This does not exclude, however, the possibility that those residues in subunits *a* or *c* or both also participate in this process. Bragg also reported that phenylglyoxal inhibited the binding of F_1 to F_0 in membrane vesicles. However, incorporation of [^{14}C]phenylglyoxal appeared to occur only into subunits *b* and *c* (19). The reason for the discrepancy with the above-mentioned results is unclear.

Treatment of *E. coli* F_0 with tetranitromethane, in contrast to that of TF_0, failed to inhibit any F_0 function (194). In both cases the modification of tyrosine residues in subunit *c* was directly demonstrated by amino acid analysis (188; K. Steffens and K. Altendorf, unpublished data).

Dissociation and Reassembly

What is the role of the single subunits of F_0 in H^+ translocation and in the interaction with F_1? Are they all required for maintaining these functions or is subunit *c* by

itself sufficient for creating an H^+ channel? Again, Kagawa's group was the first to address these questions. In 1978 Sone et al. (190) reported on the preparation of a TF_0 subcomplex, which they claimed to be composed of only the two smaller subunits (139). This preparation still exhibited passive H^+ translocation (although at a reduced rate), reconstituted TF_1F_0 ATPase activity, and ATP-$^{32}P_i$ exchange. From this the authors concluded that the largest (19-kDa) subunit is not necessary for those activities and may merely have a structural function. However, as evident from the densitogram of their SDS gel (190), the two-subunit TF_0 contained an additional polypeptide (>19 kDa) that was already present in intact TF_0 in comparable amounts (190). This protein may well be an inherent constituent of TF_0. Thus, a definitive conclusion as to the role of the third subunit has to await the identification of the genes coding for the ATP synthase subunits in PS 3.

By gel filtration in the presence of SDS, the 19-kDa-deficient TF_0 complex was further resolved (190). The smallest subunit, identified as the DCCD-binding protein, failed to translocate H^+ after incorporation into phospholipid vesicles (see also following section). Surprisingly, liposomes containing the 13.5-kDa polypeptide bound TF_1; no such reaction was observed with the DCCD-binding protein. However, as the data given for the TF_1-binding activity do not suffice for a comparison with intact TF_0 preparations, the significance of the binding efficacy and specificity cannot be judged. Moreover, the data do not exclude the possibility that the smallest subunit might also be involved in TF_1 binding in vivo. It is conceivable that potential binding sites on subunit *c* for TF_1 become accessible only when the proteolipid is in an oligomeric state and that in its turn the formation of an oligomer is dependent on the presence of the 13.5-kDa subunit. A direct proof that the isolated subunits (specifically the 13.5-kDa protein) have retained their activities would be the reconstruction of a functional TF_0 complex from isolated subunits; this has not been reported yet.

In *E. coli* the question of the role of the single F_0 subunits has been addressed by genetic as well as biochemical approaches. By analyzing *E. coli* strains that carried plasmids with various combinations of F_0 genes, Friedl et al. (55, 57) provided evidence that all subunits have to be present to form a functional F_0 complex. Strains lacking one or two of the F_0 subunits did not exhibit any H^+ translocation at all, whereas two-subunit combinations were active in rebinding F_1 to a certain extent. However, none of the reported reconstituted ATPase activities reached the value obtained with intact F_1-stripped membranes. Since these activities were insensitive toward DCCD, it is doubtful whether F_1 was bound to its physiological binding sites. Basically, these results were confirmed by several other groups who studied *E. coli* mutants with defective or missing F_0 subunits (23, 48, 107, 125, 151). These studies, however, did not exclude the possibility that the requirement for all subunits was limited to the initial in vivo assembly process: conceivably, the subunits might "survive" a resolution of the complex once they had established their native conformation. Schneider and Altendorf (167, 168) have performed a series of dissociation experiments to address this view (Fig. 4). First, subunit *b* was extracted from purified F_0. Reconstituted into liposomes, the *a–c* complex did not exhibit either H^+ translocation or F_1 binding (167). However, preincubation of the *a–c* complex with stoichiometric amounts of subunit *b* and subsequent incorporation into phospholipid vesicles resulted in a fully active F_0 complex. These findings strongly indicated that also in vitro all three subunits are required for a functional F_0 portion. (Also, Perlin and Senior [149] reported the purification of subunit *b*. They extracted washed *E. coli* membranes with Triton X-100 and obtained a purified protein by passing the soluble fraction through hydroxylapatite. However, the ability of the preparation to restore F_0 functions has not yet been demonstrated.) This view was further supported by experiments involving the complete dissociation of F_0 (168) (Fig. 5). After incorporation into liposomes,

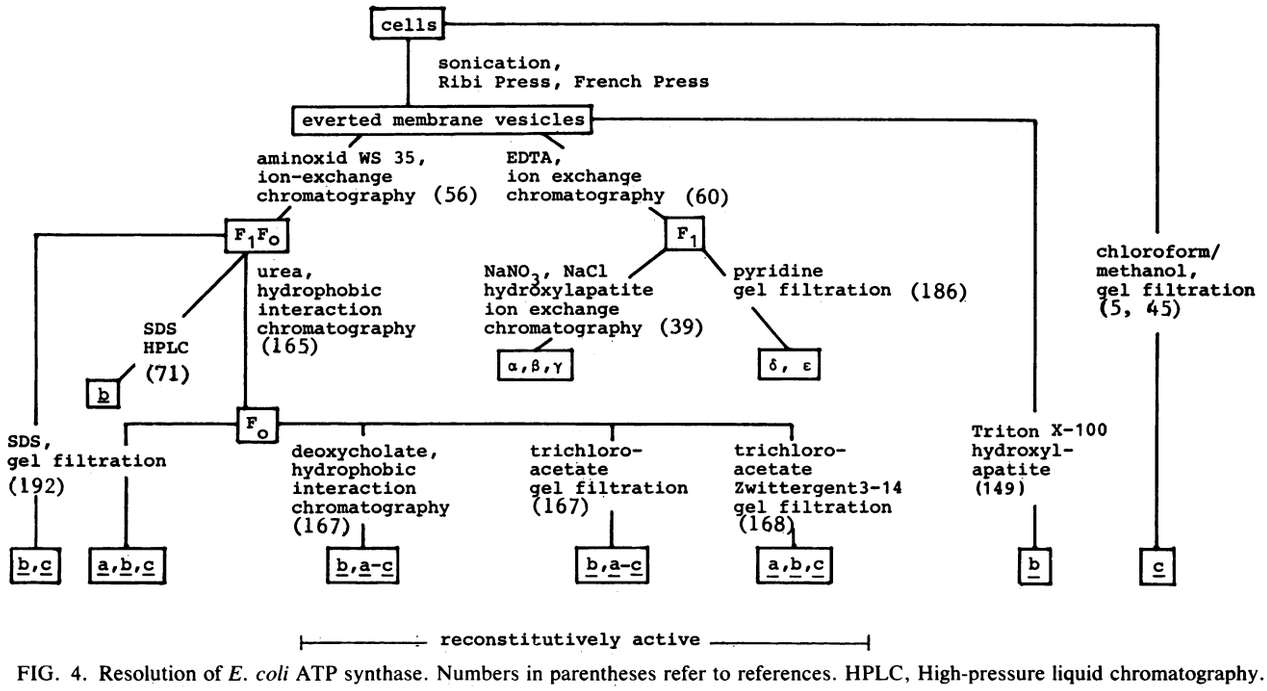

FIG. 4. Resolution of *E. coli* ATP synthase. Numbers in parentheses refer to references. HPLC, High-pressure liquid chromatography.

none of the possible combinations of subunits except a_1 b_2 c_{10} (corresponding to intact F_0) restored any F_0 function (Fig. 6). With the latter combination, both H^+ translocation and reconstituted ATPase activity were restored to almost the control values obtained with intact F_0 and were highly sensitive to DCCD. Interestingly, when subunit *c* was prepared by extraction with chloroform-methanol from whole cells (5, 45) or gel filtration of F_0 in the presence of SDS (192) (Fig. 4), reconstitution with subunits *a* and *b* failed to yield an active complex. This finding indicates that in these preparations subunit *c* is present in a conformational state that does not allow a proper interaction with subunits *a* and *b*. Although the view that subunits *a* and *b* serve to stabilize an H^+ channel built up by a subunit *c* oligomer is quite appealing, it does not rule out a more direct involvement of *a* and *b* in F_0 functions.

As to subunit *b*, the following information is at hand. Trypsin or chymotrypsin treatment of F_1-stripped membrane vesicles (73, 76, 147) or of liposomes containing F_0 (193) affected only subunit *b*, thereby abolishing the binding of F_1. H^+ translocation was fully retained. Three initial trypsin-generated cleavage products of about 16 kDa were observed which remained associated with the membrane (149, 193). These polypeptides were converted into two stable products of 12 and 8.3 kDa which have been detected by modifying Cys-21 in the membrane-embedded N-terminal region of subunit *b* by a fluorescent maleimide derivative (169, 193). These observations give rise to the notion that the N-terminal portion of subunit *b* may be required for stabilizing the proton channel. In agreement with this, modification of Cys-21 affects reassembly of a functional F_0 from isolated subunits (169). Also, effects on either assembly or functions have been reported for *E. coli* strains carrying a missense mutation in that part of subunit *b* (86–88, 151). A more direct proof might come from reconstitution experiments involving the N-terminal portion of subunit *b*.

On the basis of its primary structure the hydrophilic C-terminal part of subunit *b* has been considered to be involved in the interaction with F_1 (217). This view is supported by several lines of evidence. Subunit *b* is protected against proteolytic cleavage in the presence of F_1 (73, 154). Furthermore, anti-*b* antibodies as well as chemical modification of arginine residues disturb the interaction of F_0 with F_1 (6a, 149; B. Merrem and K. Altendorf, unpublished results). Cross-linking experiments demonstrated that subunit *b* and β of F_1 are in close proximity to each other (10). However, the hydrophilic part may also be important for the proper insertion of F_0 into the lipid bilayer. Mutants in which glycine at position 131 of subunit *b* has been replaced by aspartate are impaired in both F_1 binding and H^+ translocation (151) and in the proper assembly of a functional proton channel in vivo (88). Furthermore, F_0 isolated from trypsin-treated membranes and, therefore, lacking the C-terminal part of subunit *b* did not exhibit H^+ translocation or F_1-binding activity when incorporated into liposomes (193).

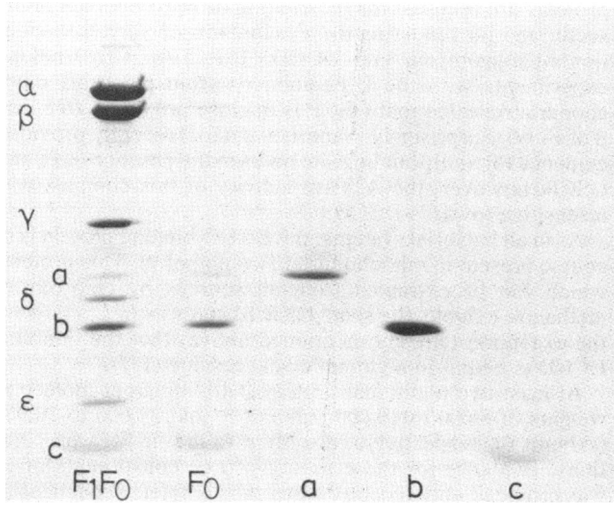

FIG. 5. SDS gel of purified preparations of *E. coli* F_1F_0, F_0, and isolated F_0 subunits. Taken from *Methods in Enzymology* (170) with permission of the publisher.

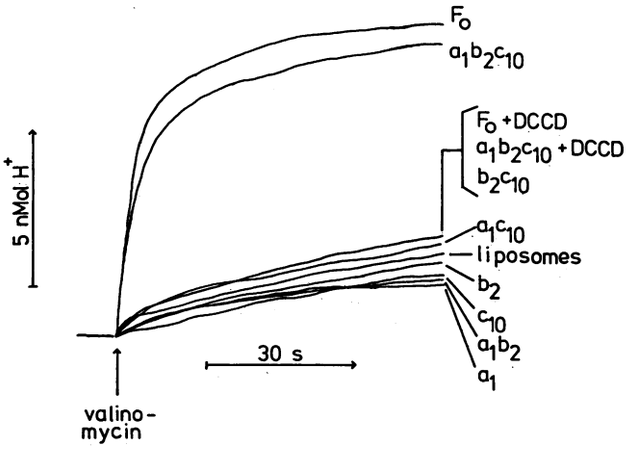

FIG. 6. H^+ translocation through *E. coli* F_0 or various subcomplexes, incorporated into liposomes. After preloading with K^+, the reaction was started by the addition of valinomycin and pH changes were monitored with a pH electrode (see text). Taken from *EMBO Journal* (168) with permission of the publisher.

Little is known about the particular function(s) of subunit *a*. Recently, indirect evidence for an H^+ translocating function of *a* has been reported by von Meyenburg et al. (214). Overproduction of subunit *a* alone resulted in the inhibition of growth and protein synthesis which could be restored by the addition of DCCD. Whether this effect is due to the overproduction per se or reflects an intrinsic property of subunit *a* remains to be established. In this context it should be noted that conserved polar residues in the C-terminal region (residues 189 to 219 and 250 to 265) of subunit *a* have been found in *E. coli* as well as in homologous mitochondrial subunits from different sources and in chloroplasts (23, 28, 30, 72, 115, 218). This observation gave rise to the notion that at least part of subunit *a* may participate in the formation of a proton wire. This view has been addressed in a recent model for the mechanism of the ATP synthase by Cox et al. (28; see last section for further discussion). Again, experimental evidence came from analysis of mutant strains. Most of the mutations resulted in the formation of a truncated polypeptide, thereby abolishing the assembly of a functional F_0 complex (23, 48). Missense mutations in which Ser-206 is replaced by leucine or His-245 is replaced by tyrosine are impaired or fully blocked in H^+ translocation, respectively (23). Surprisingly, in both cases F_1 does bind to the membrane and the resulting ATPase activity is sensitive to DCCD.

Similarities and Dissimilarities

To what degree are F_0 complexes from different bacteria comparable? Only in the case of *E. coli* has biochemical evidence on the F_0 subunits been complemented by genetic data and vice versa. The genes coding for the enzyme subunits are arranged in an operon (*unc*) that maps at 84 min on the chromosome (11). The F_0 genes are clustered in the order *uncB* (*a*), *uncE* (*c*), *uncF* (*b*) and precede the F_1 genes (Fig. 2). However, this does not appear to be a general rule for all bacterial ATP genes. Recently, Walker and coworkers (42, 206) established that the F_1 genes from two photosynthetic bacteria are not linked to F_0 genes. So far these genes have not yet been found.

Only after the genes for the PS 3 ATP synthase have been cloned and sequenced might the similarities and dissimilarities between TF_0 and *E. coli* F_0 be fully appreciated. As described in the preceding chapter, although the rates of H^+ translocation through either of the complexes are highly comparable, two kinds of subunits rather than three as in *E. coli* have been claimed to be sufficient in TF_0. In addition, unlike in *E. coli*, the 13.5-kDa protein from PS 3 alone has been reported to exhibit F_1-binding activity.

Also, the results from chemical modification studies differ in several aspects. This leaves us with the question of whether the F_0 sector in PS 3 may be structurally and functionally different from *E. coli* F_0. There is, however, accumulating evidence against this. First, the structure of TF_1 resembles that of *E. coli* F_1 (92, 93). Moreover, TF_1 and F_1 subunits are in part interchangeable (202), and, finally, *E. coli* F_1 functionally interacts with TF_0 liposomes (191a). For the time being, we therefore would postulate that not only the F_0 complex from *E. coli*, but also the F_0 complexes from other bacteria (including PS 3) are composed of three different subunits that are all required for its function.

COMPARISON WITH MITOCHONDRIAL AND CHLOROPLAST SYSTEMS

Preparations

The F_1F_0 ATP synthase has been isolated from a variety of eucaryotic mitochondria including those from fungi (156, 178, 204, 208), mammals (15, 35, 85, 112, 121, 156, 183, 196), *Xenopus laevis* (97), and sea urchins (36). However, F_0 preparations have been reported from mammalian sources only (3, 61, 64, 160, 184). All preparations except one were obtained by dissociation of a purified F_1F_0 complex with chaotropic reagents. Alfonzo et al. (3) described a preparation which was obtained from F_1-depleted submitochondrial particles (inner membrane vesicles with reverse orientation obtained by sonication) by sequential extraction with cholate and octylglucoside followed by fractionation with ammonium sulfate. Depending on the mitochondrial source and the homogeneity of the isolated F_1F_0 complex, the preparations differ in the number of polypeptides observed (see below; Table 1).

The ATP synthase from spinach chloroplasts (CF_1F_0) has been purified by Pick and Racker (150) and used for the isolation of CF_0 (129). Alternatively, CF_0 was prepared from CF_1-stripped chloroplast membranes (223). Most recently, a CF_0 preparation was obtained from lettuce by solubilization of membranes with a mixture of ammonium sulfate, octylglucoside, and cholate, followed by centrifugation of the crude extract through a sucrose gradient (99). Finally, the most active fractions were further purified by using immobilized poly(L-lysine)-deoxycholic acid (31; see also Table 1). All preparations differ in protein yield and their polypeptide composition.

Subunit Composition

With respect to their polypeptide composition, the mitochondrial F_1F_0 complexes (and the F_0 portion in particular) appear to be more complex than their bacterial counterparts. In addition to the five subunits α to ε and a special ATPase inhibitor protein (70, 152), which all belong to F_1, the F_0 sector is composed of four to six different subunits.

The most striking difference from the bacterial F_1F_0 seems to be the existence of a third distinct enzyme portion, namely, a "stalklike" structure, which connects F_1 to the membrane sector and is composed of two proteins called OSCP (oligomycin sensitivity-conferring protein) (117, 157) and F_6 (96). (In the following, we will consider these proteins as F_0 components.) Both proteins have been isolated from intact mitochondria and the amino acid sequences have been determined recently (43, 142). OSCP is a basic protein of M_r 20,967 and exhibits regions of homology to the sequence of the *E. coli* δ subunit in F_1 (142, 216, 218); the latter has been demonstrated to be essential for the binding of F_1 to F_0 (195). (It was also found that the mitochondrial δ subunit exhibits striking homologies to the *E. coli* ε subunit, but there seems to be no counterpart for the mitochondrial ε subunit in *E. coli* [216].) Also, some homology to certain parts of *E. coli* subunit *b* has been reported. Interestingly, this homology starts at position 1 of OSCP and 21 of subunit *b*, thereby indicating that OSCP lacks most if not all of the membrane-spanning region of subunit *b* (141). This is in accord with the finding that OSCP cannot be labeled with TID (9), which modifies membrane-embedded amino acid residues. Further evidence for OSCP being a functional analog of subunit *b* of *E. coli* stems from the observation that trypsinized bovine mitochondrial F_0 binds F_1, but fails to exhibit ATP-dependent H^+ translocation due to degradation of OSCP (90). F_6 is a polypeptide composed of 76 amino acids and has a molecular weight of 9,006. So far no significant homologies to other known proteins have been detected (43). Both proteins are required for the binding of F_1 to the membrane sector and for rendering the F_1 complex sensitive to inhibition by oligomycin and DCCD (157, 210). Cross-linking experiments with the F_1F_0 complex from pig heart mitochondria revealed that OSCP is in close proximity to α and β of F_1 (9). Surprisingly, Sandri et al. (161) recently provided evidence for an F_1-binding site on F_0 in the absence of F_6 and OSCP; however, the ATPase activity of this complex was insensitive towards DCCD.

As in all bacterial systems, the DCCD-binding protein is of course present in mitochondrial F_0 (subunit 9). This protein, which can be extracted from all sources by chloroform-methanol, exhibits the same basic features as in *E. coli* with the exception (already mentioned above) that the inhibitor DCCD is bound to a glutamic acid residue (177).

At least two additional proteins with apparent molecular weights of 5,000 to 10,000 (subunit 8) and 21,000 to 30,000 (subunit 6), respectively, are often found in F_1F_0 preparations. Evidence for these proteins to be constituents of F_0 was provided only recently when the complete sequences of the mitochondrial genome from different sources became available. The determined nucleotide sequence of a gene in *Saccharomyces cerevisiae* called *aap1* (118) fits well with the

amino acid sequence of an ATP synthase-associated proteolipid described recently (212). This protein with a molecular weight of 5,815 may represent subunit 8 of the F_1F_0 complex of *Saccharomyces cerevisiae* (207). On SDS gels, its apparent M_r is approximately 10,000; this discrepancy is probably caused by the fact that hydrophobic proteins usually do not migrate in SDS gels according to their true molecular weight. In addition, open reading frames exhibiting regions of homology to *aap1* have been found in *Aspergillus nidulans* (URF_x; 65) and mammalian mitochondria (A6L; 8). The function of this protein is still a matter of investigation; however, binding affinity for phosphate has been demonstrated (212). Subunit 6, encoded by another mitochondrial gene (*oli2*) in yeasts, has a molecular weight of 28,257 as determined from the DNA sequence (116). It has also been found in mammalian mitochondrial genomes and exhibits some homology with *E. coli* subunit *a* (115, 224). More strikingly, the overall hydropathy profiles of the two proteins are practically identical (115, 218). Furthermore, there is experimental evidence that a 24-kDa component of an F_0 preparation from beef heart is in fact identical to subunit 6 (91). In addition, the 24-kDa protein seems to be adjacent to OSCP as has been inferred from cross-linking studies (9, 205). The precise role of this subunit is still unknown; however, as discussed for *E. coli*, it might have a structural function. Surprisingly, an *oli2* mutant of *Saccharomyces cerevisiae* that lacked an intact subunit 6 protein still appeared to assemble an ATP synthase complex, although with greatly reduced and oligomycin-insensitive ATPase activity (25). This result would then point to a different role of homologous subunits in bacterial and mitochondrial ATP synthases. Recently, the gene products of A6L and ATPase 6 from bovine mitochondria have been isolated by chloroform-methanol extraction, thereby demonstrating their nature as integral membrane proteins (44).

Finally, three other proteins have been found in mitochondrial enzyme preparations: subunit 5 in preparations from *Saccharomyces cerevisiae* (207), F_B (158), and the so-called uncoupler-binding protein (UBP) in mammalian systems (13, 69). Subunit 5, with an apparent molecular weight of 29,000 to 35,000, is probably a contaminant, originated from another mitochondrial protein complex (140). F_B (apparent molecular weight, 11,000 to 15,000) is thought to be an integral component of the F_0 sector (158) and to be required for energy transfer between F_0 and F_1 (89). However, whether it is present in all F_0 preparations and required for H^+ translocation is still controversial. The uncoupler-binding protein, a polypeptide of 30,000 apparent molecular weight, has been characterized by its ability to react with azido-dinitrophenol (69). This protein has not been found in all preparations of F_1F_0 or F_0 reported; it is proposed to be involved in the maintenance of the local proton gradient generated by ATP hydrolysis and is not essential for passive H^+ translocation through F_0 (14).

Altogether, the data on the mitochondrial F_1F_0 system can be summarized as follows (and Table 4). (i) In general, the mitochondrial enzyme complex seems to be more complex than the bacterial system, and preparations from fungal mitochondria appear to be composed of less components than mammalian complexes. (ii) At least two subunits (6 and 8; in some cases also subunit 9) are encoded by the mitochondrial genome. (iii) OSCP, F_6, and subunits 6, 8, and 9 have been established as constituents of F_0. Uncertainties still exist about F_B and the uncoupler-binding protein. (iv) Homologies to *E. coli* subunits have been found for OSCP (to F_1-δ, F_0-*b*), subunit 6 (to F_0-*a*), and subunit 9 (to F_0-*c*).

In chloroplasts, the CF_0 sector is composed of at least three polypeptides. On SDS gels the proteins have apparent molecular weights of 15,500 to 18,000 (I), 13,000 to 16,000 (II), and 8,000 (III) (150, 220); the latter corresponds to the DCCD-binding protein. Usually (150, 199, 220), but not always (99), a fourth component (17,500; IV) has been found associated with the CF_0 complex; the gene coding for this polypeptide has been localized in the chloroplast genome (30, 72). Subunits I and III (DCCD-binding protein) have also been demonstrated to be encoded by plastid genes, whereas the gene coding for subunit II has been found in the nucleus (4, 16, 83, 220, 221).

In wheat chloroplasts, subunit I is composed of 166 amino acid residues and has an exact molecular weight of 19,001 as deduced from the nucleotide sequence (16). (It is worth mentioning that the gene coding for subunit I contains a large intron [16, 72].) The overall arrangement of the secondary structure reveals similarity to *E. coli* subunit *b*, whereas little homology can be inferred from the amino acid sequences. These findings have been confirmed recently for spinach chloroplasts (72). Remarkably, as in the case of *E. coli* subunit *b* (10, 73, 169, 176), there is evidence that CF_0-I might be present as a dimer in the chloroplast complex (201). A newly discovered subunit, IV (M_r, 27,060), may be homologous to *E. coli* subunit *a* (30, 72). This view is supported by the finding that the chloroplast gene order (IV, III, I) resembles that of *E. coli* (*a*, *c*, *b*) (30, 72). So far there is no counterpart for CF_0-II in bacteria (Table 4). Again, the DCCD-binding protein (III) from chloroplasts exhibits striking homologies to the corresponding polypeptides in other systems (177). In addition, it has been shown to function as an H^+ channel in the absence of other subunits in vitro (136, 185), lacking however the ability to interact with CF_1 (discussed in the next section). Therefore, subunits I and II have been suggested to play primarily a structural role in maintaining a functional proton channel, built up by subunit III oligomers, and to interact with CF_1 (137). Experimental evidence from cross-linking studies supports the view that subunits α, β, and γ of CF_1 are in close proximity to subunits I and II from CF_0 (155, 200, 201). In view of the differences from bacterial systems, the characterization of a well-defined functional CF_0 preparation would help to elucidate the function of the CF_0 subunits.

Reconstitution

The mitochondrial F_0 preparations available so far (all from beef heart mitochondria) exhibit both passive H^+ translocation (3, 64, 160, 184) and specific, inhibitor-sensitive binding to F_1 (3, 61, 64). The H^+-translocating

TABLE 4. Subunit equivalence in F_0 complexes

E. coli	Mitochondria	Chloroplasts	Reference(s) (and further references therein)
a	su 6	IV	30, 44, 72, 115, 218
b	OSCP	I	16, 72, 141
c	su 9	III	177
?	?	II	
(F_1-δ)	OSCP	?	142, 218
-	su 8, A6L, URF_x	?	9, 44, 65, 118
-	F_6	-	43
(-)	(F_B)	(-)	
(-)	(UBP)	(-)	

activity, however, is rather low compared with that in intact mitochondria (Table 3) (74). This could of course be a general loss of activity due to the isolation procedure. There is, however, some evidence that the inactivation of the H^+ channel is specifically triggered by the removal of F_1, as discussed by Nelson (134). At least in some cases, the rates of ATP-dependent reactions after reconstitution of F_0 with F_1 (and OSCP) are close to those obtained with submitochondrial particles or intact F_1F_0 preparations. From this it might be concluded that the F_0 preparation is basically fully active. Recent data on H^+ translocation through thylakoid CF_0 clearly demonstrated that removal of CF_1 by EDTA treatment did not affect H^+ translocation (173).

CF_0 prepared from lettuce chloroplasts interacted specifically with CF_1; in a reconstituted system in the presence of bacteriorhodopsin, the reassembled complex catalyzed light-induced ATP synthesis at a maximal rate comparable with that obtained with intact CF_1F_0 (98, 99). However, due to the poor reproduction of SDS gels, the polypeptide composition of this CF_0 preparation is difficult to verify.

Chemical Modification

F_1-depleted submitochondrial particles as well as liposomes containing the isolated F_0 complex have been used to study the effect of various group-specific reagents on F_0 functions. Modification of the glutamic acid residue in subunit *c* by DCCD resulted in the inhibition of H^+ translocation, as did modification of tyrosine and arginine residues (66, 67). Surprisingly, whereas the arginine-specific compound phenylglyoxal caused inhibition, the even more specific reagent 2,3-butanedione enhanced the rate of H^+ translocation (66, 144). Thus, it was concluded that different basic residues, thought to be located in the DCCD-binding protein, contribute to the formation of an "H^+ wire" (66). Also, diethylpyrocarbonate, which specifically modifies histidine residues under certain conditions, was reported (144) to enhance proton translocation. The site of action of this compound is still unknown. The DCCD-binding protein cannot be involved as it does not contain any histidine residue (177).

Various research groups studied the effect of different sulfhydryl-specific reagents on F_0 functions. Some of these compounds inhibited H^+ translocation; others were found to cause a remarkable stimulation (144). *N*-[^{14}C]ethylmaleimide, which caused inhibition of proton translocation, was demonstrated to modify the 30-kDa protein (3, 14, 144). Whether other proteins also became labeled, including DCCD-binding protein (144) (the protein from beef heart is the only one known to contain a cysteine residue [177]) or factor B (158), is controversial (14). Other sulfhydryl reagents such as Cu^{2+}-*o*-phenanthroline, Cd^{2+} (159, 160), and diamide (84, 224) have been claimed to abolish F_0 functions by interacting with F_B.

In the case of CF_0, only the DCCD-binding site in subunit III is well characterized (177). Some evidence for the fact that tyrosine residues are required for H^+ translocation came from reconstitution studies with the isolated subunit III (185; see below).

Reconstitution of the DCCD-Binding Protein (Proteolipid)

Several groups have reported that proton transport could be reconstituted by incorporation of the isolated proteolipid into artificial lipid membranes.

Nelson et al. (136) and Sigrist-Nelson and Azzi (185) reconstituted a proteolipid fraction from chloroplasts into liposomes and presented evidence that the proteolipid catalyzed a DCCD-sensitive passive H^+ movement (driven by bacteriorhodopsin and a K^+ diffusion potential, respectively; Table 3). However, the purity of those preparations (especially the first one) is difficult to judge, making interpretation of the data difficult. This also applies to similar work by Célis (24) on butanol-extracted proteolipid from heart mitochondria. Agarwal and Kalra (2) reported on the reconstitution of the butanol-extracted proteolipid of *M. phlei*. In addition to DCCD, the H^+-translocating activity is also affected by vanadate, making the interpretation of the data even more difficult.

Criddle et al. (33) added phospholipid vesicles containing a proteolipid fraction from yeast mitochondria to one side of phospholipid-impregnated membrane filters (Millipore Corp.); they introduced liposomes containing bacteriorhodopsin into the other compartment. They found that the proteolipid liposomes caused a progressive decrease in the photopotential, which was again fully restored by the addition of oligomycin. The proteolipid from *E. coli*, also isolated by the chloroform-methanol procedure and assayed by the same technique, failed to show any activity (33). The experimental results have been used to argue that a proteolipid fraction isolated from the mitochondrial inner membrane acts as a proton ionophore. Moran et al. (124) arrived at a similar conclusion, using the proteolipid from chloroplasts and applying the same technique. In contrast to Criddle et al. (33), they observed a decrease in the photopotential only when the proteolipid was incorporated into the same vesicles as the bacteriorhodopsin. In both cases, however, the system as well as the proteolipid fractions used are ill defined; therefore, no general conclusion about the applicability of the system and the proton-translocating activity of the proteolipid fractions can be drawn.

A more convincing piece of evidence that the proteolipid from yeast mitochondrial ATP synthase exhibits protonophoric activity has been provided by Konishi et al. (100). The proteolipid was isolated by chloroform-methanol extraction and reconstituted into liposomes. By imposing an electrical potential difference across the vesicle membrane (K^+/valinomycin), proton movement could be detected that was sensitive to oligomycin. Based on the data provided, an initial rate of H^+ uptake of about 40×10^3 ng of H^+ min^{-1} mg^{-1} could be estimated. The interpretation of the latter experiments is complicated by the unusual finding that the incorporation of larger amounts of proteolipid induced a K^+ conductance, the increase of which was also blocked by oligomycin (32).

Recently, single-channel conductance has been recorded after fusion of liposomes containing butanol-extracted yeast mitochondrial proteolipid with planar membranes (162). This is the first report on its proton conductivity at a high time resolution. At pH 2.2, corresponding to an H^+ concentration of about 10 mM, single-channel conductivity was observed. Stepwise changes in membrane current were taken as evidence that transitions, such as opening and closing, of single-ion pathways in the membrane occur. The channels operated independently and were highly selective for protons. About 10^7 protons per s passed each channel at 100-mV membrane potential and pH 2.2 (equivalent to 100 protons at pH 7.0). Interestingly, most of the proteolipid molecules were not in an active state; this might explain the low overall activity measured in most previous experiments (162). The reaction that leads to channel opening was second order as to proteolipid concentration (162).

Therefore, the formation of channels from a pool of

proteolipid molecules involves a bimolecular self-association. The reacting species might be the monomeric proteolipid; alternatively, it could be an oligomer itself (dimer or trimer). Further experiments indicated that at higher pH values the proton pathway was stabilized by the formation of larger oligomers, presumably starting from dimers. It is appealing to consider the possibility that also in vivo the 6 to 12 proteolipids in the F_0 complex represent a self-stabilizing association of dimers. It is, however, conceivable that the interaction between proteolipid subunits may not be sufficiently strong to promote the formation of a proton-conducting proteolipid oligomer in biological membranes. Finally, as pointed out by Schulten and Schulten (175), the possibility should not be disregarded that nonspecific water channels might form spontaneously in the planar membrane at low pH. Even so, the experiments reported by Schindler and Nelson offer some evidence that isolated proteolipid from eucaryotic sources, in contrast to that from *E. coli* or PS 3, may form a proton channel in the absence of other F_0 subunits. A possible explanation for this difference might be that, with one exception (100), all active chloroplast and mitochondrial proteolipid preparations were obtained by the butanol extraction procedure, whereas the proteolipid from *E. coli* (133) and PS 3 (190) cannot be solubilized in butanol. Conceivably, butanol might be a more favorable solvent than chloroform-methanol for the retainment of a functionally active conformation of the proteolipid.

Since the pH-induced transition of conductance reported by Schindler and Nelson (162) was only found in the presence of sufficiently high proteolipid concentrations, we would like to close this section with a word of caution against the use of abnormally high concentrations of hydrophobic proteins. It has been reported recently that overproduction of subunit *a* in vivo leads to an enhanced proton permeability of the membrane which could be reversed by DCCD (214). Such an effect has not been detected yet with normal concentrations of subunit *a* and may be due to an increased interaction between *a* subunits via their hydrophobic domains. This, in time, would favor the formation of a multimeric state which after incorporation into the membrane could exhibit proton-conducting activity. A similar explanation might also apply to the unusual observation by Criddle et al. (32; mentioned above) in which large amounts of mitochondrial proteolipid in liposomes gave rise to K^+ conductance.

OPEN QUESTIONS, PERSPECTIVES, AND MODELS

Although in reconstitution experiments the proteolipid from mitochondria seems to be able to form a proton channel by itself (see above), it may well be that under in vivo conditions other F_0 subunits are required for H^+ transport. Specifically, recent experimental evidence indicates that at least subunit *a* is a likely participant in H^+ translocation and does not play only a structural role in F_0. Cain and Simoni (23) reported on the isolation and characterization of missense mutations located in the C-terminal segment of subunit *a* from *E. coli*. In this case F_0-mediated proton translocation is impaired without altering the assembly of the F_1F_0 complex. Interestingly, these mutations lie in the region where conserved amino acid residues have been found (23, 28, 30, 72). Further evidence of the importance of the C-terminal end of subunit *a* for H^+ translocation stems from a separate series of experiments. The replacement of Gly-9 to Asp in subunit *b* results in the inhibition of H^+ translocation and in a reduced capacity to bind F_1 (86, 151). Partial F_1F_0 functions are, however, restored by an additional mutation in subunit *a* replacing Pro-240 by Ala or Leu (102). Recently, von Meyenburg et al. (214) suggested that subunit *a* from *E. coli* has protonophoric activity based upon in vivo experiments in which subunit *a* was synthesized at very high levels. While the physiological difficulties arising from extensive overproduction of an integral membrane protein and the indirect nature of their measurements allow alternative interpretations, the implication of subunit *a* being involved in proton translocation as suggested in that report remains intriguing.

By contrast, it is unlikely that the *b* subunit provides residues participating in H^+ translocation: these should be located within the short hydrophobic N-terminal region which shows only limited interaction with the other subunits (75, 167). However, it should be pointed out that chemical modification of Cys-21 of subunit *b* results in reduced H^+-translocating activity, leaving the F_1-binding capacity untouched (169).

Anyway, the discussion on the mechanism of proton translocation should no longer concentrate on subunit *c* oligomers alone, but should also include at least part of subunit *a*. In this way, sufficient polar and charged residues from amino acid side chains become available to construct a network of hydrogen bonds across the membrane.

As proton conduction occurs not only in F_0 of the ATP synthase, but also in bacteriorhodopsin (197) and redox-mediated H^+ pumping such as cytochrome *c* oxidase (222), this process is of general biological interest. However, from the discussion above it is quite clear that any mechanism of H^+ translocation remains speculative since basic information such as subunit function, subunit interaction, and orientation of the proteins within the membrane still need to be established. Nevertheless, speculations, hypotheses, and models have been very useful in paving the way to a better understanding of the system in question.

Both for the F_0 complex from ATP synthases and for bacteriorhodopsin, a proton-conducting pathway (proton wire), effectively a channel from the site of H^+ release to the aqueous phase, has been proposed. Since such a channel must be specific to H^+, it cannot just be an aqueous pore through the membrane. To be ion selective, the latter would have to be supplemented with some sort of an H^+-specific filter. Speculations have focused on models in which a network of hydrogen bonds spans the membrane and conducts protons across it. Several possible types of networks (37, 38, 40, 54, 131, 132) and channels of immobilized water molecules have been presented. Together with the nature of the channel formed by alamethicin (53), they have already been discussed in some detail in a previous review (80). More recently, based on the conductance observed for single-proton channels formed by the proteolipid from yeast mitochondria (162), Schulten and Schulten (175) have presented a simple algebraic expression for the resistance predicted for a hydrogen-bonded network model and compared their results with the experimental observations. They arrived at the interesting hypothesis that the conduction involves a series of bound water molecules and perhaps amino acid side chains. In this model, the rotation of the groups involved in the conductance process should be rather fast. This implies that the hydrogen bonds between the conductor groups are weak and that amino acid side chains with aromatic rings are not involved.

Based on structural considerations, genetic data, and biochemical evidence, Cox et al. (28, 29) have proposed a model in which, by analogy with flagellar motion, a rotation

of the *b* subunits within a core of nine *c* subunits together with the associated δ, ε, and γ subunits of F_1 drives conformational changes that result in ATP synthesis. In the more recent version (28), a new structure for subunit *a* was deduced from the amino acid sequence with only five transmembrane helices instead of six or seven as proposed previously (73, 179, 218). It was suggested that the conserved amphipathic helix 4, which was so far considered to be localized outside the membrane, traverses the phospholipid bilayer. It was further hypothesized that certain conserved residues within this segment together with Asp-61 of subunit *c* form a proton pore. Thus, the rotation of the inner complex consisting of subunits *a* and *b* from F_0 and γ, δ, and ε from F_1 relative to the outer components consisting of subunit *c* oligomers would allow successive interactions of helix 4 with the conserved acidic residue of each of the *c* subunits. However, both the new and the old versions of the model are in disagreement with data obtained from TID-labeling experiments, which indicate that subunit *a* is also accessible from the lipid phase (75). This makes it unlikely that subunits *a* and *b* are located inside a core formed by 6 to 12 copies of subunit *c*. Rather, the results call for an arrangement of a highly asymmetric nature (75, 81) (Fig. 7).

In comparison to the rotational model described by Cox et al. (28), the arrangement of the F_0 subunits given in Fig. 7 provides an interesting alternative. In this case it is suggested that the subunit *c* oligomer rotates within the membrane relative to subunits *a* and *b*. H^+ translocation occurs at the interface between subunits *a* and *c* by a mechanism similar to that proposed by Cox et al. (28). However, experimental evidence for a rotational movement within the F_0 complex is still missing.

Another mechanism of proton translocation, which involves movement of a negatively charged amino acid side chain, has been developed by Boyer (18). Such a mechanism requires conformational changes in F_0. For the F_1 ATPase, conformational changes do occur upon energization of the membrane and during ATP hydrolysis and synthesis (for review, see reference 213). Thus, it is quite conceivable that structural changes in F_1 are accompanied by or even coupled to conformational changes in F_0. In a recent series of elegant experiments on beef heart submitochondrial particles, Penefsky (145) provided convincing evidence that the tight binding of ATP to the high-affinity catalytic sites in F_1 was drastically reduced when either oligomycin or DCCD had reacted with the F_0 part of the F_1F_0 complex. This observation was interpreted to mean that conformational changes induced by the binding of these inhibitors to F_0 are relayed over some distance to the catalytic site in F_1. The author suggests (145) that relay of similar conformational changes brought about by protonation/deprotonation of one or several key amino acid residues in F_0 is involved in displacing tightly bound ATP from a catalytic site during ATP synthesis. In agreement with this notion, Penefsky (146) has shown that upon generation of an electrochemical gradient of protons, a dissociation of tightly bound ATP occurs.

Similar experiments have been reported by Hatefi and co-workers (120). They observed that in submitochondrial particles or isolated F_1F_0 complex, ATP-induced fluorescence change of F_1-bound aurovertin could be inhibited by DCCD or oligomycin. Since aurovertin binds to the β subunits of F_1, the data suggest that a conformational change in F_0 caused by DCCD is communicated to the β subunits of F_1. Other evidence for a conformational change within mitochondrial F_0 includes the observation (182) that F_0 in the absence of F_1 acts as a voltage-gated channel and that F_0 in submitochondrial particles exhibits two different conformational states depending on the $\Delta\tilde{\mu}_{H^+}$ applied (101). In bacteria the evidence for conformational changes in F_0 is rather scarce. However, based on mutant analysis, Fillingame et al. (47, 49) and Hoppe et al. (78) suggested that the inhibition of F_1 ATPase activity by DCCD is not directly caused by inhibiting H^+ translocation through F_0, but rather by a conformational change in F_0, which distorts the structure of F_1.

In this context it should be pointed out, as has already been done by Sebald and Hoppe (177), that the reactivity of Asp-61 in subunit *c* towards DCCD is difficult to reconcile with a participation in the H^+ translocation process. DCCD is not only very bulky but also very hydrophobic and, therefore, probably reacts from the lipid phase. One solution to this problem could be that Asp-61 is not directly involved in the H^+ translocation process but rather contributes to the stabilization of a certain conformation of F_0 active in H^+ translocation. Modification of Asp-61 by DCCD would neutralize the negative charge, giving rise to a conformational change in F_0, the result of which is a nonconducting proton wire. In a rotational model the modification of Asp-61 could lead to inhibition of movement of subunit *c* oligomer relative to subunits *a* and *b*.

Some evidence against a conformational change of F_0 has been reported. Recently, intact mitochondria from *Neurospora crassa* and thylakoids from *Pisum sativum* have been labeled with a carbene generated from [^{125}I]TID by a single ultraviolet laser pulse (219). The analysis of the two isolated proteolipids revealed no difference in either labeling pattern upon energization. The authors arrived at the conclusion that the proteolipid oligomer forms a rigid and

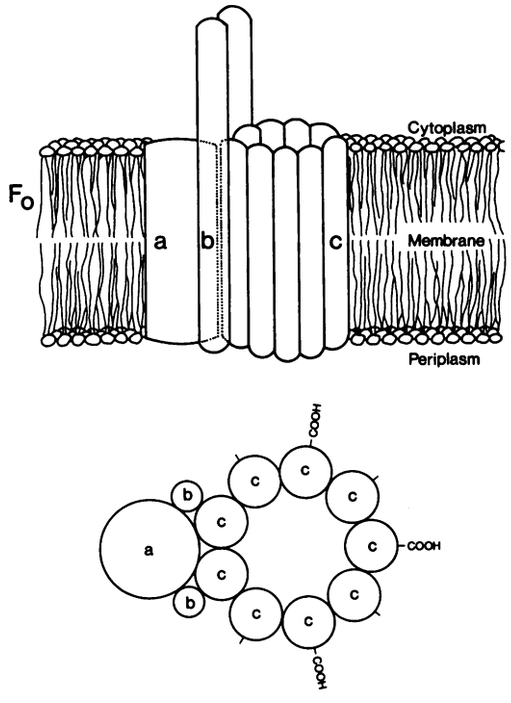

FIG. 7. Model for F_0 subunit arrangement. (Top) Putative arrangements of subunits *a*, *b*, and *c* of the *E. coli* F_0 complex in the membrane. (Bottom) Top view of the *E. coli* F_0 complex indicating that the carboxyl group (Asp-61, DCCD-binding site) is in contact with the lipid phase.

compact core and interpreted this to mean that the conformation of F_0 might be subjected to only minor alterations upon inhibitor binding and ATP synthesis. Since only one subunit of F_0 has been analyzed so far, this conclusion is rather premature.

In summary, there is indirect evidence for conformational changes in F_0 but direct proof is still lacking. What evidence there is makes it difficult to distinguish between conformation changes or rotational movement within F_0. Membrane-permeating photoreactive probes and covalent attachment of reporter groups, e.g., fluorescent labels or spin labels, might help to solve this problem.

The existence of 6 to 12 copies of proteolipid per F_0 has prompted many researchers in the field to propose models about the structural arrangement of the subunits in F_0. One simple possibility could be that a ring of monomers as suggested for alamethicin (53) exists. In this case a β-barrel structure, a common structural element of many proteins (128), may be adopted by the interaction of possible β-sheet segments in subunit *c*. As already pointed out, this arrangement is well suited for rotational movement within F_0. The question remains of whether all proposed 10 copies of subunit *c* are participating in an H^+ channel at the same time. The observation that maximal inhibition of H^+ translocation by DCCD was achieved when only a fraction of subunit *c* had become modified (177) has been interpreted to mean that only a fraction of the *c* subunits is either always active or required for function (47, 155). However, it should be pointed out that in the rotational model the modification by DCCD of 1 of 10 copies of subunit *c* should be sufficient to completely block H^+ translocation.

To distinguish between trimers or a ring of nine protomers of subunit *c* and to elucidate the arrangement of subunits *a* and *b* within F_0, structural analysis of F_0 or F_1F_0 crystals is necessary. In addition, site-directed mutagenesis might help to clarify which amino acid residues are promising candidates for constructing a proton wire through F_0.

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